Increased activity associated with reduced sensitivity of acetylcholinesterase in organophosphate-resistant greenbug, *Schizaphis graminum* (Homoptera: Aphididae)

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Abstract: The activity and sensitivity of acetylcholinesterase (AChE, EC 1.1.1.7) from an organophosphate-susceptible (OSS) and three resistant (OR-0, OR-1 and OR-2) strains of the greenbug (*Schizaphis graminum*) were biochemically compared. All three resistant strains displayed higher frequencies of individuals with respect to both increased activity of AChE toward the model substrate acetylthiocholine (ATC) and reduced sensitivity of AChE to inhibition by paraoxon as compared with the OSS strain. Kinetic study indicated that AChE from the OR-0, OR-1 and OR-2 strains had 3.3-, 2.7- and 2.3-fold, respectively, lower affinity, but 1.5-, 2.2- and 2.0-fold, respectively, higher catalytic activity toward ATC than AChE from the OSS strain. Significantly increased activity of AChE in the resistant strains was also confirmed by non-denaturing polyacrylamide gel electrophoresis, and appeared to be associated with the increase of general esterase activity. Inhibition kinetics revealed that AChE from the OR-0, OR-1 and OR-2 strains was 2.1-, 2.2- and 2.7-fold less sensitive to inhibition by paraoxon than that from the OSS strain. The study suggested that both qualitative and quantitative modifications of AChE had evolved in the resistant strains and were likely to significantly enhance the overall resistance level in greenbugs.

Keywords: *Schizaphis graminum;* insecticide resistance; resistance mechanism; altered acetylcholinesterase; enzyme kinetics

1 INTRODUCTION

Organophosphate and carbamate insecticides exert their neurotoxic effects by inhibiting acetylcholinesterase (AChE, EC 1.1.1.7), a critical enzyme involved in nerve impulse transmission.[1,2] Consequently, decreased sensitivity of AChE to inhibition by these insecticides has been implicated in insecticide resistance in many insect and other arthropod species. To date, at least 30 arthropod species have been reported to have an insensitive AChE conferring or contributing to organophosphate and/or carbamate resistance. These species include 25 summarized by Fournier and Mutero[3] and others such as the greenbug (*Schizaphis graminum* (Rond.),[4,5] peach-potato aphid (*Myzus persicae* Subz), tobacco aphid (*M. nicotianae* Blackman),[6] pear psylla (*Cacopsylla pyricola* Forster),[7] and lesser grain borer (*Rhizopertha dominica* (F.)).[8] Molecular studies in several resistant insect species indicate that the decrease of AChE sensitivity to inhibition by organophosphates and/or carbamates is due to mutation(s) of the AChE gene.[9–11] Such mutations result in structural modifications of the enzyme, which often shows qualitatively modified enzyme properties, including reduced sensitivity of the enzyme to inhibition by organophosphate and/or carbamate insecticides.

Although less well documented as compared with the qualitative modifications of AChE, altered AChE associated with increased AChE activity has been reported in several insect species including housefly (*Musca domestica* L.),[12] green rice leafhopper (*Nephotettix cincticeps* (Uhl.)),[13] fruitfly (*Drosophila melanogaster* Meig.),[14] and *R. dominica*.[8] The increased AChE activity may reflect either a quantitatively increased production of the enzyme with unchanged catalytic properties or a qualitatively modified enzyme with a higher catalytic efficiency. Fournier *et al*[14] demonstrated a strong correlation between AChE content in the central nervous system and susceptibility to insecticides in *Drosophila*. Larger amounts of AChE overexpressed in the insect

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Contract/grant sponsor: Kansas Agriculture Experiment Station. Contract/grant number: H498
(*Received 9 March 1998; revised version received 20 June 1998; accepted 3 August 1998*)

nervous system may function as a scavenger of insecticide molecules, leading to increased tolerance without altering the basis of synaptic transmission.

Reduced sensitivity of AChE to inhibition by paraoxon was recently reported as an insecticide-resistance mechanism in two organophosphate-resistant strains of the greenbug *S. graminium* by Siegfried and Ono.[4,5] In this paper, we report: (1) frequency distributions of greenbugs with respect to the sensitivity of AChE to inhibition by paraoxon and specific activity of AChE in an organophosphate-susceptible and three resistant strains; (2) kinetic and electrophoretic comparisons of AChE among the four greenbug strains; and (3) relationship between AChE and general esterase activities in each of the four strains. Our studies indicated that reduced sensitivity of AChE to inhibition by paraoxon was associated with increased catalytic activity to substrate in all three resistant strains. Such a combination of both qualitative and quantitative modifications associated with organophosphate resistance has been poorly documented in insect and other arthropod species.

2 MATERIAL AND METHODS

2.1 Greenbug strains

An organophosphate-susceptible colony (OSS) was initially collected from sorghum plants in the greenhouse at Kansas State University (KSU), Manhattan, Kansas. A marginally resistant colony (OR-0), possessing an insensitive AChE, was provided by John C. Reese at KSU. Two highly resistant strains (OR-1 and OR-2), possessing Types I and II esterases, respectively, were provided by Blair D Siegfried at the University of Nebraska, Lincoln, NE. The sources and characteristics of these two strains have been previously described in detail.[15] All strains were maintained in the stems of one-to-two month old sorghum plants supplied with water in two-liter flasks at *c.* 23°C, and the stems were replaced about every five days.

2.2 Chemicals

Acetylthiocholine iodide (ATC), bicinchoninic acid (BCA) solution, 5,5′-dithio-bis (2-nitrobenzoic acid) (DTNB), fast blue B salt (*O*-dianisidine, tetrazotized), α-naphthol, α-naphthyl acetate, paraoxon (diethyl *p*-nitrophenyl phosphate, 90% pure), sodium dodecylsulfate (SDS), and Triton X-100 were purchased from Sigma Chemical Co. (St. Louis, MO). Acrylamide, bovine serum albumin, *N*,*N*′-methylene-bis-acrylamide, *N*,*N*,*N*′,*N*′-tetra methylethylenediamine and Tris [1,1-bis(hydroxymethyl)-2-hydroxyethylamine] were purchased from Bio-Rad Laboratories (Hercules, CA).

2.3 Enzyme microassay in individual greenbugs

A single apterous adult of greenbug was homogenized in phosphate buffer (0.1 M; pH 7.5; 180 µl) containing Triton X-100 (3 ml litre^{-1}). The homogenate was centrifuged at 15 000*g* for 15 min at 4°C, and the supernatant was then used as the enzyme source. AChE activity and its inhibition by paraoxon were determined according to the method of Ellman *et al*[16] as modified by Zhu *et al*[10] using ATC as substrate. Briefly, the reaction mixture, consisting of ATC (0.5 mM), DTNB (0.04 mM), paraoxon (5 µM) and enzyme preparation (50 µl) was prepared in a final volume of 150 µl with phosphate buffer (0.1 M; pH 7.5). Absorbance was recorded at 405 nm for 5 min at 23°C with a V_{max} kinetic microplate reader (Molecular Devices, Menlo Park, CA). The remaining AChE activity was determined against the control that was performed in parallel but lacked paraoxon.

For general esterase assays, 15 µl of the same enzyme preparation that was used for the determinations of the AChE activity and sensitivity was incubated in a final reaction volume of 150 µl containing α-naphthyl acetate (0.27 mM) at 37°C for 30 min. The reactions were stopped by adding fast blue-SDS solution (50 µl)[17] and the absorbance determined at 600 nm 15 min later using the microplate reader.[18]

2.4 AChE kinetics

Fifty apterous adults were homogenized in ice-cold phosphate buffer (0.1 M; 7.5; 1 ml) containing Triton X-100 (3 ml liter^{-1}). After the homogenates had been centrifuged at 15 000*g* for 15 min at 4°C, the supernatants were diluted 5-fold with phosphate buffer (0.1 M; pH 7.5) and the diluted enzyme preparations used for enzyme kinetics as previously described.[19] The AChE activities at 12 concentrations (8 mM to 3.9 µM) of ATC were determined, based on the reaction for 2 min, whereas the initial velocity was determined based on the reaction for 30 s at 23°C with the microplate reader at 405 nm. Michaelis constant (K_m) and maximal velocity (V_{max}) values were determined by Hanes transformations.[20]

Inhibition kinetics of paraoxon was performed based on the method of Main[21] with some modifications.[22] To approach first-order kinetics for the inhibition, relatively high concentrations of paraoxon, low temperature (23°C) and short incubation time (2 min) were used. The inhibition reaction was stopped by the addition of ATC and the 20-fold dilution of the inhibitor. The remaining AChE activity was determined for 2 min under the same conditions. Bimolecular reaction (k_i), affinity (K_a) and phosphorylation (k_p) constants were calculated according to Main.[21]

2.5 Assays of protein contents

Protein contents of the enzyme preparations were determined according to Smith *et al*[23] using bovine serum albumin as standard. The measurement was performed with the microplate reader at 560 nm.

2.6 Electrophoretic analysis of AChE

Non-denaturing polyacrylamide gel electrophoresis was carried out based on the method as previously described by Zhu and Clark[19] using a Penguin P8DS dual-gel electrophoresis system (Owl Scientific, Inc., Woburn, MA) coupled with a cold-water circulating system. Fifteen apterous adult greenbugs were homogenized in phosphate buffer (0.1 M; pH 7.5; 50 µl) containing Triton X-100 (3 ml liter^{-1}). After centrifugation at 15 000*g* for 15 min at 4°C, 20 µl of supernatant (equivalent to six greenbugs) was mixed with 10 µl sample buffer and the mixture was loaded into each well of the gel. The gel (4 and 6% acrylamide in stacking and separating gels, respectively) was run at a constant voltage of 100 v for 2 h. Both the gels and electrophoresis buffers contained Triton X-100 (1 ml liter^{-1}). The AChE bands were visualized by incubating the gels in staining mixture overnight at room temperature.[24]

3 RESULTS

3.1 Frequency distributions of greenbugs with respect to the sensitivity and specific activity of AChE

Figure 1 shows the frequency distributions of greenbugs with respect to the sensitivity of AChE to inhibition by paraoxon in an organophosphate-susceptible (i.e. OSS) and three resistant (i.e. OR-0, OR-1 and OR-2) strains. All three resistant strains displayed a higher frequency of individuals with higher percentage of remaining activity of AChE than that of the OSS strain. AChE from many individuals in the OR-0 and most individuals in the OR-1 and OR-2 was significantly less sensitive to inhibition by paraoxon than that of the OSS strain. The mean of the remaining AChE activity in the presence of 5 µM paraoxon in the OR-0, OR-1 and OR-2 strains was 1.2-, 1.4- and 1.5-fold, respectively, higher than that in the OSS strain (Table 1). Besides the reduced sensitivity of AChE to inhibition by paraoxon in the resistant strains, frequency distributions of greenbugs with respect to the higher AChE activity in three resistant strains were also higher than that in the OSS strain (Fig 2). The mean of the AChE activity in the OR-0, OR-1 and OR-2 strains was 1.9-, 2.8- and 2.0-fold, respectively, higher than that in the OSS strain (Table 1). Further, AChE activity of all three resistant strains was less homogeneous than that of the OSS strain.

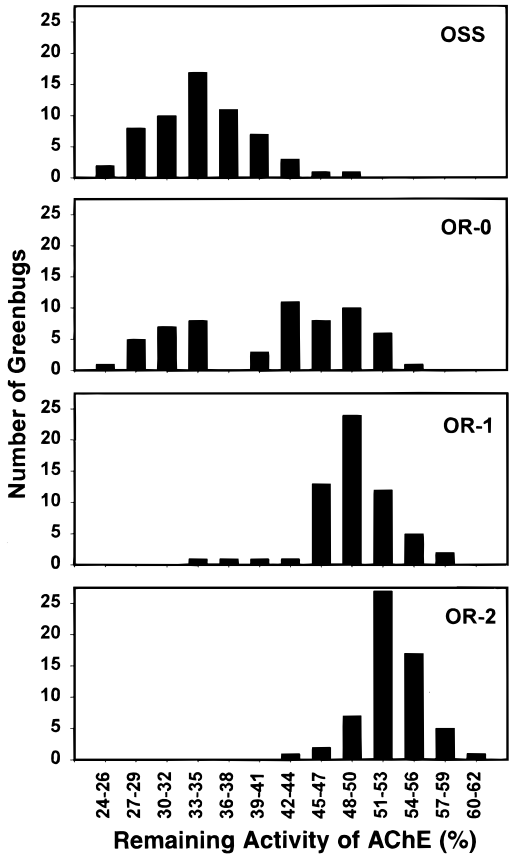

Figure 1. Frequency distributions of greenbugs with respect to the sensitivity of AChE to inhibition by paraoxon in the organophosphate-susceptible (OSS) and resistant (OR-0, OR-1 and OR-2) strains. The percentage remaining activity of AChE was individually determined in 60 greenbugs for each strain using the AChE microassay.

Table 1. Comparison of activity and sensitivity of acetylcholinesterase to inhibition by paraoxon in individuals of organophosphate-susceptible and resistant strains of greenbugs[a]

Strain	*Remaining activity* (%)	*Specific activity* (*nmol min*$^{-1}$ *mg*$^{-1}$)
OSS	34.6 (±4.8)a	18.9 (±4.6)a
OR-0	41.4 (±8.2)b	36.7 (±15.3)b
OR-1	49.0 (±4.1)c	52.6 (±19.9)c
OR-2	52.8 (±3.0)d	38.5 (±10.9)b

[a] Results are the mean (±SD) of 60 single-aphid determinations. Means within columns followed by the same letter are not significantly different ($P > 0.05$; Duncan's[31] multiple range test by using SAS program[32]).

3.2 Kinetic and electrophoretic analysis of AChE

Effects of substrate (i.e. ATC) concentrations on AChE were examined in all four strains of greenbug. The overall patterns of the enzyme responses to the change of substrate concentration were similar among the three resistant strains but were different from that of the OSS strain (Fig 3), suggesting that AChEs in the resistant strains were kinetically different from that in the OSS strain. The kinetic study indicated that AChEs from the OR-0, OR-1 and OR-2 strains had 3.3-, 2.7- and 2.3-fold, respectively, lower affinity (i.e. higher K_m values) to the substrate ATC than that from the OSS strain (Table 2). In contrast, the catalytic activities of AChE toward ATC in the OR-O, OR-1 and OR-2 strains were 1.5-, 2.2- and 2.0-fold higher (i.e. higher V_{max} values) than that in the OSS strain. Significant increase of the AChE activity in the resistant strains

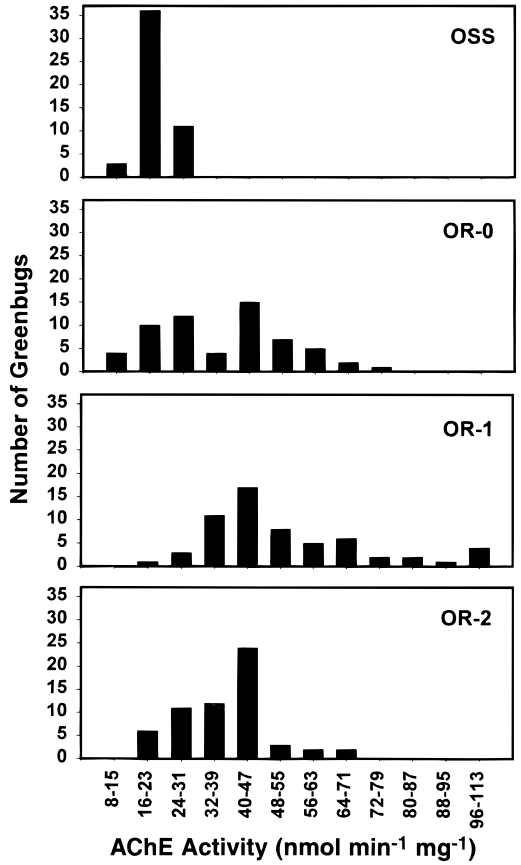

Figure 2. Frequency distributions of greenbugs with respect to the specific activity of AChE hydrolysing acetylthiocholine in (○) organophosphate-susceptible (OSS) and resistant (OR-0, OR-1 and OR-2) strains. The activity was individually determined in 60 greenbugs for each strain using the AChE microassay.

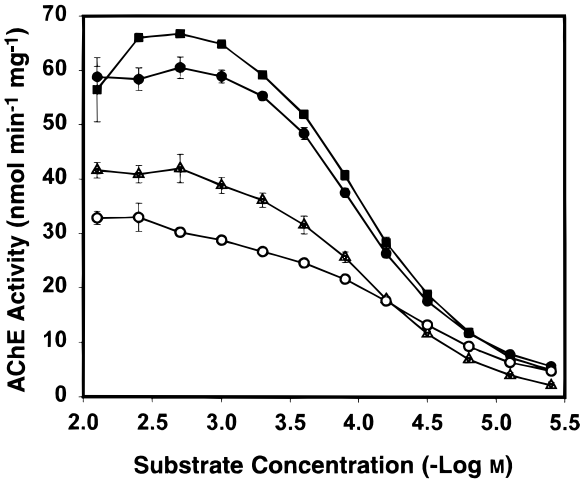

Figure 3. Effect of substrate (i.e. acetylthiocholine) concentration on the AChE activity at 23°C and pH 7.5 in the organophosphate-susceptible (OSS, ○) and resistant (OR-0, △; OR-1, ■, and OR-2, ●) strains of greenbugs. Each point represents the mean of four determinations ($n = 4$). Vertical bars indicate standard deviations of the mean.

was also confirmed by non-denaturing polyacrylamide gel electrophoresis, showing remarkable increases of the stain intensity in all resistant strains when gels were stained for the AChE activity (Fig 4).

Inhibition kinetics of AChE indicated that AChE from the OR-0, OR-1 and OR-2 strains was 2.1-, 2.2- and 2.7-fold less sensitive to inhibition by paraoxon than that from the OSS strain. These results agreed well with the results in the frequency distribution study with respect to the sensitivity of AChE to paraoxon (see Fig 1). Apparently, the reduced sensitivity of AChE in the resistant strains was not caused by the change of the phosphorylation constant (k_p) but was probably due to the decrease of the affinity between AChE and the organophosphate molecule. The reduced k_i values were strongly corre-

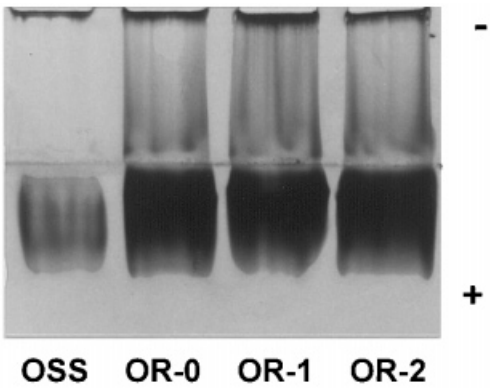

Figure 4. Non-denaturing polyacrylamide gel electrophoresis of AChE extracted from the organophosphate-susceptible (OSS) and resistant (OR-0, OR-1 and OR-2) strains of greenbugs. Each lane was loaded the same amount of sample equivalent to six apterous adults, and the AChE was stained for its activity using acetylthiocholine as substrate.

Table 2. Kinetic analysis of acetylcholinesterase hydrolysing acetylthiocholine and inhibition by Paraoxon in organophosphate-susceptible and resistant strains of greenbug[a]

Strain	K_m[b] (μM)	V_{max} (nmol min^{-1} mg^{-1})	$K_a \times 10^6$ (M)	k_p (min^{-1})	$k_i \times 10^{-5}$ (M^{-1} min^{-1})	Ratio of k_i (S/R)
OSS	22.8 ± 2.4a	31.3 ± 0.3a	2.13 ± 0.36a	1.44 ± 0.15a	6.82 ± 0.56a	—
OR-0	74.4 ± 4.5b	46.1 ± 1.1b	4.55 ± 1.72ab	1.47 ± 0.42a	3.32 ± 0.35b	2.1
OR-1	60.6 ± 5.8c	69.8 ± 2.9c	4.82 ± 1.00ab	1.45 ± 0.23a	3.04 ± 0.22bc	2.2
OR-2	52.9 ± 2.5d	62.9 ± 1.4d	7.95 ± 4.75b	1.89 ± 0.79a	2.55 ± 0.38c	2.7

[a] Results are the mean (± SD) of four determinations. Means within columns followed by the same letter are not significantly different ($P > 0.05$; Duncan's[31] multiple range test by using SAS program[32]).

[b] K_m and V_{max} are affinity constant and maximum velocity, respectively, of the enzyme hydrolysing acetylthiocholine as substrate; K_a, k_p and k_i are affinity, phosphorylation and bimolecular reaction constants, respectively, of the enzyme inhibition of paraoxon.

lated to the increased K_a values in all three resistant strains.

3.3 Relationship between the AChE and general esterase activities

Microassays for both the general esterase and AChE activities in individual greenbugs revealed a positive correlation between general esterase and AChE activities in all three resistant strains (Fig 5). Because non-AChE esterases virtually cannot use ATC (a rather specific substrate for AChE) as substrate, it is unlikely that the higher activity of general esterase activity contributed to the higher AChE activity in the resistant strains. This contention was also supported by the lack of such correlation in the OSS strain.

4 DISCUSSION

The present study indicated that all three organophosphate-resistant strains of greenbugs possessed an altered AChE with decreased sensitivity to inhibition by paraoxon, and decreased affinity and increased catalytic activity toward the model substrate ATC. Our results agreed well with those reported by Siegfried and Ono[4,5] with respect to the involvement of insensitive AChE in conferring organophosphate resistance in greenbugs. Because AChE from the resistant strains had both 2- to 3-fold reduced affinity to ATC (i.e. increased K_m values) and reduced sensitivity to inhibition by paraoxon (i.e. decreased k_i values) as compared with AChE from the susceptible strain, it was clear that all three resistant strains possessed a qualitatively altered AChE. Interestingly, AChE from the resistant greenbug strains also showed 1.5- to 2.2-fold higher catalytic activity toward ATC (i.e. increased V_{max} values). Because the altered AChE in the resistant strains actually had lower affinity to ATC, the degree of increased catalytic activity of AChE in the resistant strains might have been underestimated in this study because the decreased AChE affinity to ATC is likely to reduce the overall activity. Significant decrease of the sensitivity to inhibition by paraoxon and affinity to ATC, and increase of the catalytic activity toward ATC in the resistant strains strongly suggested that AChE in resistant greenbugs had been involved in both qualitative and quantitative modifications.

Fournier *et al*[14] demonstrated that there was a strong correlation between the amount of AChE expressed in the central nervous system and susceptibility to insecticides in *Drosophila*, which suggested

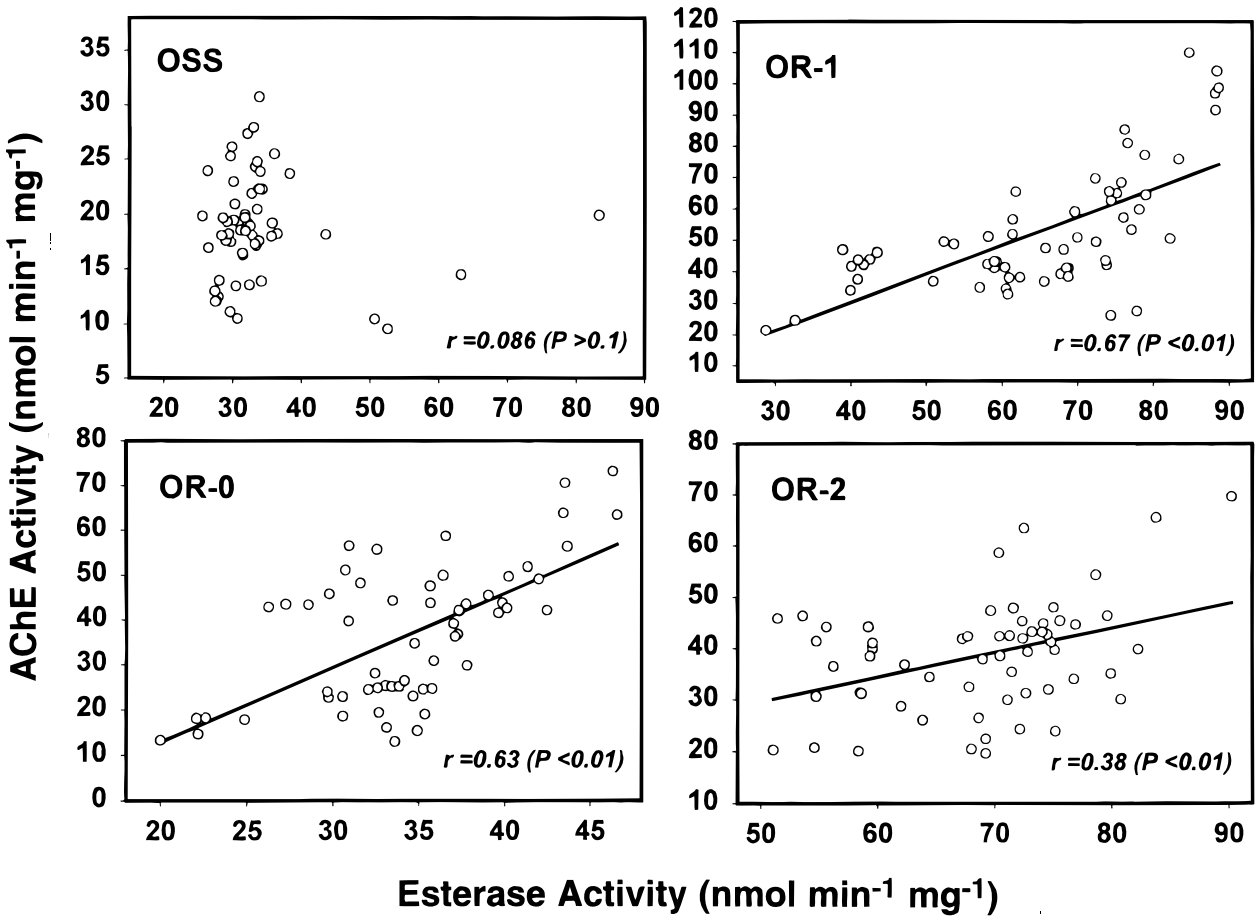

Figure 5. Relationship between the general esterase and AChE activities in the organophosphate-susceptible (OSS) and resistant (OR-0, OR-1 and OR-2) strains of greenbugs. In each strain, 60 apterous adults were assayed for both the specific activities of general esterases and AChE using α-naphthyl acetate and acetylthiocholine, respectively, as their substrates. The increased activity of AChE in the resistant strains was positively correlated with the increased general esterase activity ($P < 0.01$).

that increased AChE activity could result in insecticide resistance. A similar phenomenon has also been found in other insect species such as lesser grain borer,[8] housefly,[12] and green rice leafhopper.[13] However, it is not feasible to define the significance of increased AChE activity in contributing to organophosphate resistance in greenbugs due to the involvement of insensitive AChE and increased general esterase activity which were previously reported as predominant resistance mechanisms in greenbugs.[4,5,25,26]

Nevertheless, our previous study showed that OR-0, OR-1 and OR-2 strains were 1.6, 32.1, and 41.6, respectively, more resistant to parathion than the OSS strain.[27] Because the general esterase levels in the OR-0 strain were virtually the same as that of the OSS strain, the marginal resistance in the OR-0 strain was apparently due to the modified AChE with increased activity and reduced sensitivity to organophosphates. Our results suggested that the modified AChE alone was not sufficient to cause a high degree of resistance in insects; the latter would only arise if other effective resistance mechanisms such as enhanced metabolic detoxification, reduced penetration, and/or increased sequestration coexisted.[28]

Indeed, the presence of a less-sensitive AChE could dramatically enhance the overall resistance level when other resistance mechanisms coexist. In peach-potato aphids, the presence of an insensitive AChE in an esterase-based resistant clone brought the resistance level from 9- to 122-fold to pirimicab, and from 6.3 to 180-fold to triazamate.[6] Such a synergistic effect of the site insensitivity mechanism on the overall resistance level has also been noticed in western tarnished plant bug (*Lygus hesperus* Knight)[28] and tobacco budworm (*Heliothis virescens* F).[29] Because of the synergistic nature of different resistance mechanisms, it is logical to expect that the significance of modified AChE in contributing to the overall resistance in the OR-1 and OR-2 strains is much greater than the levels of insensitivity that we determined in this study, due to the involvement of the 2- to 3-fold increase of the general esterase activity as a predominant resistance mechanism.

The existence of a positive correlation between the general esterase and AChE activities in the resistant strains of greenbugs is particularly interesting. Such a correlation could mean the co-selection of AChE and other esterases by insecticides especially if increased AChE is a factor contributing to the overall resistance.[30] Genetically, two genes encoding these enzymes may be closely linked or their expression could be regulated by the same factor(s). However, the normal general esterase level associated with the increased AChE activity in the OR-0 strain implied that other mechanisms might also contribute to or modify such relationship. Further study is required to understand the genetic and molecular basis of the altered AChE associated with organophosphate resistance, mechanisms of reduced sensitivity and increased activity of AChE, and possible interactions between the modified AChE and increased general esterase activity in conferring organophosphate resistance in greenbugs.

ACKNOWLEDGMENTS

The authors thanks Drs BD Siegfried (University of Nebraska at Lincoln) and JC Reese (Kansas State University) for providing the greenbug strains used in this study, and Drs R Shufran and GE Wilde (Kansas State University) for reviewing an early version of this manuscript. This work was supported by Kansas Agricultural Experiment Station (Project No. H498), and the manuscript is contribution No. 98-331-J from the Kansas Agricultural Experiment Station, Kansas State University. Greenbug voucher specimens (No. 083) are located in the KSU Museum of Entomological and Prairie Arthropod Research, Kansas State University, Manhattan, KS 66506, USA.

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